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Resolution and binding site determination of D,L-thyronine by high-performance liquid chromatography using immobilized albumin as chiral stationary phase. Determination of the optical purity of thyroxine in tablets*

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Abstract Human and bovine serum albumin bound to silica or aminopropyl silica were used as chiral stationary phases (CSPs) D,L-Thyronine, D,L-tryptophan, *N*-benzoyl-D,L-phenylalanine, D,L-warfarin and D,L-benzoin could be resolved on these CSPs using a mobile phase of 0 05 M phosphate buffer, pH 7 0 The capacity factor of D-thyronine was higher than that of L-thyronine The resolution of D,L-thyronine was completely lost by the presence of bilirubin in the mobile phase, but only little affected by caprylate By contrast, the resolution of D,L-tryptophan was not affected by bilirubin, but lost by the presence of caprylate These results are consistent with binding of D-thyronine to the bilirubin binding site and L-tryptophan to the caprylate binding site in albumin, respectively, and suggests that such "displacement chromatography" can be used for the determination of binding sites The optical purity of D-thyroxine in tablets was determined indirectly after de-iodination by catalytic hydrogenation

Keywords *Albumin, chiral liquid chromatography, thyronine, thyroxine, protein binding, determination in tablets*

Introduction

The thyroid hormone L-thyroxine (3,3′,5,5′-tetraiodo-L-thyronine, L-T4) exerts various physiological effects in mammals Thus, L-T4 increases the basal metabolic rate, causes a decrease in the serum cholesterol level and plays an important role in the control of connective tissue [1, 2]

The corresponding D-enantiomer of the hormone similarly reduces serum cholesterol and causes suppression of the thyroid stimulating hormone (TSH), but has no effect on the basal metabolic rate [1, 3–5] The therapeutic use of D-T4 necessitates an optical purity in excess of 99% to avoid side effects due to the L-enantiomer [6]

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The T4 enantiomers have previously been resolved by reversed-phase HPLC, either after derivatization with tert-butyloxy-L-leucine *N*-hydroxysuccinimide esters to produce diastereomeric dipeptides [7], or after complexation with other amino acids (e g L-phenylalanine or L-proline) and cupric ions present in the mobile phase [8] Recently, a chiral stationary phase (CSP) containing L-proline and cupric ions bound on silica was used for the direct resolution of several (iodo-)thyronines [9]

A variety of CSPs for resolution of enantiomers has been described in the literature and has recently been reviewed [10–12] One of these CSPs, containing albumin, has proved valuable for the resolution of several aromatic chiral compounds, including tryptophan, kynurenines and *N*-benzoylated amino acids [13–15] The resolution is due to the preferential binding of one enantiomer to the protein, which probably carries a number of binding regions/sites with binding capacity for a range of drugs and some endogenous compounds (for reviews, see refs 16 and 17) Albumin is known to bind L-T4 and presumably assists in the transport of the hormone within the body [18] Because of our interest in protein immobilization, we found it worthwhile to investigate the possible use of the albumin CSPs prepared in our laboratory for resolution of enantiomers of (iodo-)thyronines

The present paper describes simple procedures for immobilization of human and bovine albumin to both silica and aminopropyl silica The resolution of D,L-thyronine (D,L-T) is based on the selective binding of D-thyronine to albumin at the bilirubin site (cf refs 16 and 17) The chromatographic resolution of D- and L-thyronine was used for determination of small amounts of L-T4 in D-T4 tablets after de-iodination by hydrogenation

Materials and Methods

Chemicals

D,L-Thyronine (T-1501), *p*-nitrophenyl acetate, and bovine serum albumin, fraction V (BSA) were obtained from Sigma (St Louis, MO, USA) Human serum albumin, fraction V (HSA I), D,L-tryptophan (puriss), L-thyroxine sodium salt (98%), caprylic acid, and 5% palladium on barium sulphate (puriss) were from Fluka AG (Buchs SG , Switzerland) A 20% solution of HSA (HSA II) was obtained as a gift from Nordisk Gentofte A/S (Copenhagen, Denmark) HSA III (trocken, reinst) was from Behringswerk (Marburg (Lahn), FRG) This HSA was found to react as quickly with *p*-nitrophenyl acetate as reported for essentially fatty-acid-free HSA (cf ref 19) Hydrogen was purchased from Dansk Ilt og Brint (Copenhagen, Denmark) D-Thyroxine (art No 24201) was from Henning (Berlin, FRG) L-Amino acid oxidase in solution from Crotalus durissus (102792) was purchased from Bohringer Mannheim GmbH (Mannheim, FRG) The silica used for the chromatographic columns was Nucleosil R Si 300-5 (71243) from Macherey-Nagel (Duren, FRG) and 3-aminopropyl triethoxysilan was from EGA-Chemie (Steinheim, FRG) Bondelut C-18 (100 mg ml^{-1}) cartridges were from Analytichem International (Harbor, CA, USA) Solvents were either distilled or analytical grade *N*-benzoyl-D,L-phenylalanine was prepared by the method described in ref 13 Warferin sodium salt was from Nygaard & Co (Norway) Benzoin and bilirubin were from Merck (Darmstadt, FRG)

Instrumentation

The chromatographic system used consisted of an LDC Constametric Model III pump equipped with a Rheodyne 7125 valve with a 20 μl loop, an albumin CSP column, and a

Kontrol Uvicon LCD 725 variable wavelength detector connected to a Spectra-Physics SP-4270 integrator

Preparation of the CSP

Silica (2 g) was added to a solution of 5 ml 3-aminopropyl-triethoxysilan in 45 ml toluene in a flask equipped with a reflux condenser Without stirring, the mixture was heated overnight at 90°C on an oil bath The following day, the mixture was filtered with suction on sintered glass, washed with 100 ml ethanol and dried in an oven at 100°C This aminopropyl silica was packed in a 125 × 4 6 mm i d stainless steel column by conventional slurry packing techniques A solution of 4 mg ml^{-1} albumin in 0 05 M sodium phosphate buffer (pH 5 0 or pH 7 0) was pumped through the column at a flow-rate of 1 ml min^{-1} and the eluate monitored at 280 nm The break-through point appeared after approx 20 min The albumin solution was allowed to pass through the system for another 20 min before being replaced with an albumin-free buffer, which was applied for approx 100 min, until the baseline was stable at the wavelength used for analysis (254 nm) The amount of bound protein was determined by spectrophotometry at 280 nm of the starting albumin solution applied to the column and the collected eluate from the column

Standard solutions

The (iodo-)thyronines (5–10 mg) and tryptophan were dissolved in 0 01 M sodium hydroxide and appropriately diluted (1–0 001 mM)

De-iodination and sample preparation

Five D-T4 tablets, each containing 1 mg D-T4, were suspended in 5 00 ml 0 01 M sodium hydroxide in a 10 ml test tube with screw cap and rotated for 15 min The suspension was centrifuged and 1 00 ml of the supernatant was transferred to another test tube The catalyst (20–40 mg 5% palladium/barium sulphate) was added and the reaction mixture was placed in a well ventilated hood while a gentle stream of hydrogen was passed through the mixture for 1 h Then, 5 ml 0 05 M sodium phosphate buffer (pH 5 5) was added and the sample mixture applied to a Bondelut C-18 cartridge washed in advance with 2 ml 50% methanol in buffer followed by 3 ml pure buffer After addition of the sample, the sorbent was washed with 10 ml 0 05 M phosphate buffer (pH 5 5) followed by 2 ml 50% methanol in water This latter fraction was collected in a 25 ml flask and evaporated to dryness *in vacuo* The residue was dissolved in 500 µl 0 01 M sodium hydroxide and subjected to HPLC on albumin CSPs Elution was performed with 0 05 M phosphate (pH 7 0) at a flow-rate of 1 0 ml min^{-1}, unless otherwise noted

L-Amino acid oxidase treatment of D,L-thyronine

A solution of 1 ml 0 1 mM D,L-thyronine in 0 05 M phosphate (pH 6 0) was treated at 25°C with 1 µl of the enzyme solution (as supplied) and aliquots were subjected to HPLC analysis on the CSP after 0, 10, 35, 105 min and approx 18 h The first eluting peak decreased from 50 to 47, 45, 39 and 2%, respectively, of the total peak area, and was accordingly assigned to the L-thyronine

Hydrolysis of p-*nitrophenyl acetate in the presence of albumin*

A 0 045 mM solution of HSA III was prepared from 150 mg protein dissolved in

50 00 ml 0 05 M Tris–HCl, pH 8 0 This solution (1 00 ml) was mixed with 1 00 ml of 0 01 M sodium hydroxide as blank or 1 mM D,L-thyronine or D,L-tryptophan in 0 01 M sodium hydroxide Then 1 00 ml of a 0 01 mM solution of *p*-nitrophenyl acetate (*p*-NpOAc) in 0 05 M Tris–HCl buffer, pH 8 0 was added and the mixture quickly transferred by suction into the cell of a Shimadzu 265 spectrophotometer The absorbance at 400 nm of the resulting mixture (pH 8 2) was recorded as a function of time Rate constants (k) were calculated using the equation for a second order reaction

$$\frac{1}{[\text{HSA}]_0 - [p\text{-NpOAc}]_0} \ln \frac{[p\text{-NpOAc}]_0\,[\text{HSA}]}{[\text{HSA}]_0\,[p\text{-NpOAc}]} = k\,t$$

Displacement experiments

The compounds, caprylic acid, warfarin, or bilirubin were added to the mobile phase in concentrations ranging from 0 01 to 10 mM Chromatograms of 0 1 mM solutions of D,L-tryptophan and D,L-thyronine were recorded when the background absorbance became stable

Results and Discussion

Characteristics of the CSP

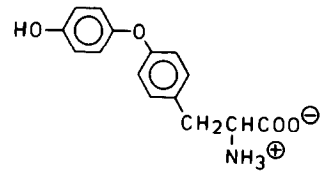
BSA and HSA could both be used as CSPs after immobilization on aminopropyl silica or underivatized silica With both types of silica approx 100 mg albumin could be adsorbed per column containing approx 1 5 g of silica (Table 1) The CSPs were tested with D,L-tryptophan and D,L-thyronine D,L-Thyronine was resolved (Fig 1) without organic modifier throughout the pH range investigated, i e between pH 3 3–7 0, and showed a slight increase in selectivity (α) with increasing pH By contrast, D,L-tryptophan was only resolved above pH 5 5

It might have been expected that the albumins would exhibit different properties, depending on whether albumin was immobilized on anionic silica or cationic aminopropyl silica However, both types of silica gave similar chromatographic results (Table 1) when tested with D,L-tryptophan or D,L-thyronine, independent of whether HSA or

Table 1
Immobilization of HSA and BSA on silica and aminopropyl silica Amount of albumin bound on a 125 × 4 6 mm i d column and chromatographic parameters (k' and α) of D,L-tryptophan and D,L-thyronine, using 0 05 M phosphate, pH 7 0, as mobile phase

	Albumin (mg)	Tryptophan k'_L	Tryptophan k'_D	Tryptophan α	Thyronine k'_L	Thyronine k'_D	Thyronine α
Silica*							
HSA II	60	0 33	0 24	1 4	0 81	1 01	1 3
BSA	140	0 87	0 39	2 2	1 41	1 84	1 3
Aminopropyl silica†							
HSA II	162	0 84	0 28	3 0	0 61	1 02	1 7
HSA II	138	1 42	0 38	3 7	0 55	0 84	1 5
HSA I‡	30	0 81	0 34	2 4	0 55	1 19	2 2
HSA I‡	15	0 48	0 19	2 5	0 32	0 74	2 3

*The column was loaded at pH 5 0, using 0 05 M phosphate
†The column was loaded at pH 7 0, using 0 05 M phosphate
‡The mobile phase was 0 05 M phosphate pH 6 0



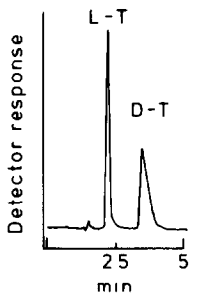


Figure 1
Resolution of D,L-thyronine on HSA (HSA II) immobilized on aminopropyl silica, packed in a 125 × 4 6 i d mm column, using 0 05 M phosphate pH 7 0 as mobile phase

BSA was used In general, the resolutions obtained with albumins adsorbed on aminopropyl silica were better than those obtained using underivatized silica Therefore, HSA II adsorbed to aminopropyl silica was used as CSP for the remainder of this study

D,L-Thyronine gave a rectilinear standard curve with a correlation coefficient better than 0 99 using areas, but the retention volume of the D-enantiomer, in particular, decreased at high concentration, which ranged from 0 001 to 1 mM The relative standard deviation of the retention volume was 4% for the D-enantiomer and 1% for the L-enantiomer The detection limit was 0 001 mM All solutions injected were made up in 0 01 M sodium hydroxide due to low solubility of thyronine at neutral pH This is believed to shorten the life of the column Baseline resolution of D,L-thyronine was achieved with the first ca 50 injections The column life-span could be extended somewhat by reloading albumin, although the amount which was immobilized the second time, was considerably less (10–20 mg) than the first time (ca 100 mg) The column could be used continuously for about 200 h, and the shelf-life was a few months at 4°C

The ability to resolve other chiral compounds was also tested using D-T4, L-T4, and some compounds which have previously been reported [13] to be resolved with albumin as CSP (i e D,L-tryptophan, D,L-benzoin, *N*-benzoyl-D,L-phenylalanine and D,L-warferin) Except for T4 all these compounds were well resolved (Table 2) D- and L-T4 showed no resolution and were only eluted after addition of propanol to the mobile phase, 15 and 30% propanol gave k' values of 9 and 2, respectively

Table 2
Effect of caprylic acid on the resolution of enantiomers on a chiral stationary phase containing HSA The mobile phase was 0 05 M phosphate pH 7 0 (A) or the same buffer containing 0 01 M caprylic acid (B)

	A			B		
	k'_1	k'_2	α	k'_1	k'_2	α
D,L-Tryptophan	0 52	0 75	1 4	0 49	0 49	1
N-benzoyl-D,L-phenylalanine*	0 48	1 02	2 1	0 29	0 29	1
D,L-Benzoin	1 76	2 31	1 3	0 90	0 90	1
D,L-Thyronine	0 81	1 09	1 3	0 69	0 94	1 4
D,L-Warferin*	1 61	2 14	1 3			

*The mobile phase contained 10% *n*-propanol

Because only racemic thyronine was available, the order of elution for the thyronine enantiomers was established after enzymatic degradation of racemic thyronine with L-amino acid oxidase as described in Materials and Methods The chromatograms clearly showed that the first eluting component gradually disappeared with time Furthermore, authentic L-T4 and D-T4 were de-iodinated with hydrogen using palladium on barium sulphate as catalyst This reaction is known to yield thyronine with retention of the optical activity [6] The resulting compounds co-chromatographed with the first and second peak of D,L-thyronine, respectively Thus it is concluded that L-thyronine elutes before D-thyronine on this albumin CSP This order of elution contrasts with the one observed for D,L-tryptophan, where the D-form elutes before the L-form, and appears to be the first example of a free D-amino acid binding stronger than the corresponding L-form to albumin

Studies on the binding site

L-Tryptophan and L-T4 as well as medium chain fatty acids like caprylic acid and a number of drugs, are known to share the same binding region in albumin [16, 17]

The presence of 0 1 mM caprylic acid in the mobile phase at pH 7 0, abolished the resolution of D,L-tryptophan, while the presence of up to 10 mM caprylic acid had little effect on the capacity factors and the resolution of D,L-thyronine (Table 2) The fact that caprylic acid affects the resolution of D,L-thyronine only to a small extent compared with the total loss of resolution for D,L-tryptophan, *N*-benzoyl-D,L-phenylalanine, and D,L-benzoine may be explained as follows either D-thyronine binds stronger to the region shared by the other compounds tested, or D-thyronine also binds to another region in albumin The latter explanation seems to be preferred because D,L-thyronine, compared with caprylate and D,L-tryptophan, had little effect on the rate of acetylation of albumin by *p*-nitrophenyl acetate (Fig 2) This reaction is reportedly [19] catalysed by binding of the *p*-nitrophenyl acetate to the same site which binds D,L-tryptophan and caprylate D,L-Tryptophan caused partial inhibition of the reaction while caprylic acid caused almost complete inhibition at the same concentration Figure 2 shows that some inhibition takes

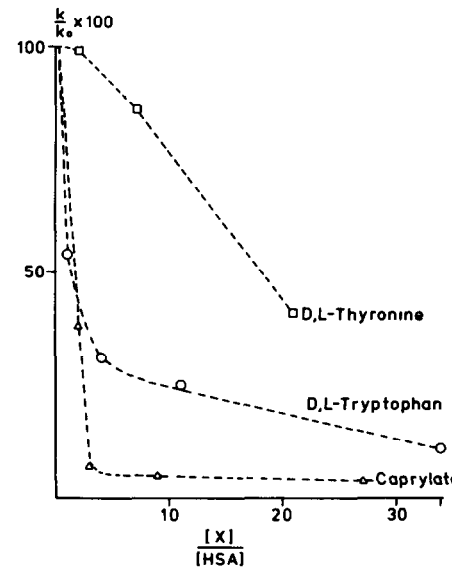


Figure 2
Inhibition of the specific acetylation of HSA (HSA III) in the presence of various compounds (X) [HSA] = $1\,6 \times 10^{-5}$ M, [*p*-NpOAc] = $3\,7 \times 10^{-6}$ M, $k_0 = 4 \times 10^3\ s^{-1}\ M^{-1}$, pH 8 2 (0 05 M Tris–HCl)

place with D,L-thyronine in large excess over HSA However, the present work does not allow any conclusions to be drawn about which of the enantiomers of thyronine might bind to the site, because the pure enantiomers were not available for study

Warfarin, which is believed to have a binding site different from that of tryptophan and caprylate (cf ref 16), was also shown not to affect the resolution of D,L-thyronine at concentration of 1 mM warfarin in the mobile phase The bilirubin binding site is assumed (cf ref 16) to be different from the binding sites of tryptophan, caprylate and warfarin Bilirubin caused a total loss of the resolution of D,L-thyronine at low concentration (0 01 mM) By contrast, the resolution of D,L-tryptophan was unaffected under the same conditions Thus, it appears that D-thyronine binds with high stereoselectivity to the bilirubin binding site

The method used here to identify the binding site of an enantiomer may be generally useful Provided compounds with a known binding site on a protein are available, their effect, when added to the mobile phase, on the chromatographic resolution of the enantiomers on a column of the immobilized protein, may indicate the binding site for one or both of the enantiomers Conversely, if the binding sites of the enantiomers are known, the effect of the additives to the mobile phase may yield information on the possible binding sites of the additives

Determination of the optical purity of D-T4 in tablets

D,L-T4 could not be directly resolved with the albumin CSPs used here However, the enantiomeric composition of T4 could be determined after de-iodination by hydrogenation to thyronine with the same enantiomeric composition [6]

$$T4 + 4\ OH^- + 4\ H_2 \rightarrow T + 4\ I^- + 4\ H_2O$$

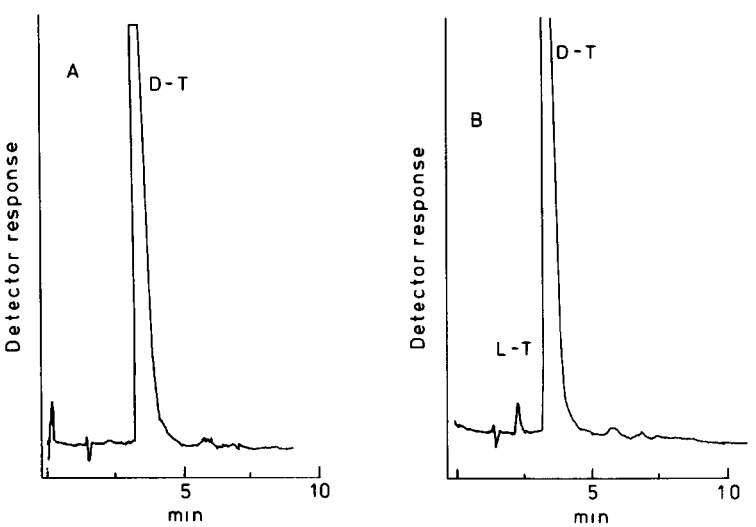


Figure 3
Determination of small percentages of L-T4 in D-T4 tablets by chromatography of samples following catalytic de-iodination (A) The content of L-thyronine in the sample was below the limit of detection (0 5%) (B) The same sample as in A after spiking with L-thyronine yielding a mixture of 1% L-thyronine and 99% D-thyronine

Table 3
Determination of the optical purity of standard mixtures of L-T4 and D-T4 after de-iodination to L-T and D-T

Sample		Optical purity % Calc	Found	cv% (n = 4)
1	L–T	0 74	0 63	7 4
	D–T	99 26	99 37	
2	L–T	0 80	0 75	7 6
	D–T	99 20	99 25	

After de-iodination the samples were applied to a reversed phase sorbent to remove free iodide, which otherwise would interfere with L-thyronine in the chromatographic determination In Table 3 the results from the determination of standard mixtures of L-T4 and D-T4 after de-iodination to L-thyronine and D-thyronine, are shown Figure 3 shows chromatograms of the sample obtained from D-T4 tablets and the same sample after spiking with L-thyronine The optical purity of D-thyronine and, consequently, of the parent compound D-T4 was higher than 99 5%

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